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*THE TRANSFER OF LEARNED BEHAVIOR FROM TRAINED TO UNTRAINED RATS BY MEANS OF BRAIN EXTRACTS, I**

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A number of recent investigations have suggested the possibility that ribonucleic acid may play an important role in the recording of memory in the central nervous system. This speculation was supported particularly by Hydén *et al.*,[1–3] who found differences in base ratios between RNA from the brains of trained rats and RNA from untrained rats. In addition, experiments on planarians[4, 5] showed that the retention of a conditioned response by a regenerated tail section could be suppressed by ribonuclease, and suggested that the effects of learning might be transferred by cannibalism of trained worms. A re-examination of this finding, however,[6] demonstrated that what was transferred seemed to be a general activating effect rather than a specific learned response.

Despite the inconclusive outcome of these early experiments, two groups were led to attempt a transfer of learned responses in the vertebrate brain, making use of an extract containing RNA; both groups have reported positive results. The first of these investigations, by Fjerdingstad, Nissen, and Røigaard-Petersen[7] showed that an intracisternal injection of an extract from the brains of rats trained to run down the lighted alley of a maze facilitated the learning of the same task by recipient rats. While this result indicates an important difference between experimental and control groups, the fact that learning in rats is facilitated by their being fed yeast RNA,[8] as well as the demonstration of a general sensitization factor in

Planaria, makes it imperative that transfer should be demonstrated by some measurement other than speed of learning. The subsequent studies by Babich *et al.*[9] successfully demonstrated the transfer of learned responses without retraining. Their extract, in which RNA was presumed to be the active ingredient, was obtained from the brains of rats trained to run to a foodbox in response to the click of a pellet dispenser. This extract was injected intraperitoneally into untrained rats, who tended to give the same response, without any reward being administered. The possibility that this result might be due to a general activating effect was controlled in a later experiment,[10] in which one group was trained to run in response to the click, while a second group was trained to respond to a light; both associations transferred selectively. Interspecies transfer, from hamsters to rats, has also been demonstrated by the same technique.[11]

A second case of interspecies transfer was reported earlier by Ungar and Oceguera-Navarro.[12] The donor animals were rats, adapted so as not to startle at the sound of a hammer dropping on a steel plate. The adaptation curve for mice, injected with a brain extract from the conditioned rats, was found to be strikingly facilitated. Although again lacking evidence for specificity, this study is significant in that the investigators, having no particular preconception as to the nature of the active substance in their extract, devoted more attention to its characterization, concluding that a peptide-type material was involved.

The series of studies to be reported here have, in part, run a parallel course to those outlined above, but have resulted in a significantly different conclusion as to the nature of the information-carrying molecule. This work was influenced in part by the theoretical speculations of Szilard,[13] Rosenblatt,[14] and others, that memory involves a process of synaptic modification, e.g., by means of an adhesive molecule[15] suitably coded to match the membranes of particular types of presynaptic and postsynaptic neurons. In a theoretical analysis (to be published later) of the most plausible properties for such a molecule, based on requirements of solubility, transportability, and information content, one of us (F. R.) was led to propose a polypeptide with a molecular weight of about 4000. An alternative hypothesis, which attributes the transfer to a genetic induction mechanism,[16, 17] also merits consideration. Two preliminary experiments by our group have already been reported,[18] demonstrating that although the Babich technique seems to produce a transfer of learned behavior, the extract is not inactivated by ribonuclease, and that contrary to previous reports,[9] an appreciable quantity of proteinaceous material is present in the extract.

In all, ten experiments have now been completed, with the following objectives: (1) to confirm the previous finding that learned behavior is transmissible by means of a brain extract, with suitable controls for general activity effects, and with automatic recording of data to provide a standard objective assay techique; (2) to explore the range of behavior susceptible to such transfer, and to demonstrate its specificity to the particular stimuli and tasks on which the donor animals are trained; (3) to attempt a preliminary chemical characterization of the substance responsible for transfer, and in particular to evaluate the conjecture that this substance may be RNA; (4) to estimate the relative distribution of the active substance between the soluble and particulate fractions of the brain; (5) to examine a number of factors, including duration of training, dosage, and use of individual versus pooled brain

extracts, which might influence the strength of the effect. This paper will describe methods and techniques of data analysis, and will summarize results obtained from the pooled data of all ten experiments. A more detailed analysis of results from separate experiments will be presented in the following paper.

Methods.—Subjects were female albino rats, supplied by Holtzman, weighing 190–210 gm, and about 90 days old. A total of 748 rats were used, including 15 "extras" used for matching properties of control and experimental groups, and four who died during the experiments. Of the remaining 729 rats, 242 were donors and 487 were recipients of brain extracts.

Experiments 1 and 2: These experiments have been described in more detail elsewhere.[18] The methods of Babich *et al.*[9] were employed for both training and extraction procedures. The learned task was to run to a food cup in the corner of a Skinner box in response to the click of the pellet dispenser, from a designated starting position (across the box, facing away from the cup). A response was counted if the rat's nose came within a prescribed distance of the cup (2 cm in expt. 1, and over the edge in expt. 2) within a given time limit. Responses were timed by an observer with a stopwatch, all tests being run "blind." Donor rats were trained for 5 days with 45-mg Noyes pellets as reinforcement (up to 200 pellets per session, administered on an intermittent schedule). Recipients were given two preconditioning sessions before injection, with ten unreinforced stimuli. Beginning 4 hr after injection, they were given a series of five unreinforced tests (five stimuli in each) spaced at 4-hr intervals. These were followed by two reinforced sessions of five stimuli each. Two scores were obtained for each rat: (1) total number of responses within 5 sec of a stimulus on the unreinforced tests; (2) number of responses within 2 sec of a stimulus on all seven tests.

The extracts used in these experiments will be referred to as "RNA extracts," although they are shown to contain other substances as well as RNA. To obtain this extract, the donor rats were killed by decapitation, and the brains homogenized in a 50% mixture of phenol and saline. The homogenate was centrifuged at 8,000 *g* for 20 min at 0–4°C, the aqueous phase collected, and magnesium chloride added to bring the concentration to 0.1 *M*. Two vol of cold ethanol were added, and the mixture was left for 30 min before centrifuging at 35,000 *g* to collect the precipitate. The precipitate was vacuum-dried, and taken up in 1 ml of saline per rat, for injection. All injections in this and following experiments were done intraperitoneally. Eight such extracts were prepared (four in each experiment) and each was injected into ten recipient rats, as follows: (*A*) and (*E*) are RNA extracts from ten untrained donors in each case; (*B*), extract from the cerebral sections of the brains of ten trained donors; (*C*), extract from the whole brains of ten donors, plus residual brain portions from (*B*); (*D*), half of extract (*C*) incubated with ribonuclease; (*F*), trained cerebellar extract, from 20 rats; (*G*), trained cerebral extract from another ten donors; (*H*), half of extract (*G*) incubated with ribonuclease. Spectrophotometric data and a Sephadex G-50 column fractionation were used to compare the RNase-incubated extracts (*C* and *G*) with known samples of RNA, and confirmed that no more than trace amounts of residual RNA could have remained. A Folin-Ciocalteu test[19] revealed a protein or polypeptide concentration of 1.1 mg/ml.

Experiment 3: A standard Scientific Prototype Skinner box, equipped for automatic counting of bar-pressing responses, was employed. Twenty-two donors were given 5 days of training with food reinforcement (30-min sessions), being advanced as quickly as possible to a fixed ratio 1:5 schedule. The 20 best donors were used for preparation of the extracts, which was done by the same technique as in experiments 1 and 2. Forty recipients were used, with 2 days of starvation, each getting two 15-min sessions in the box, without reinforcement, prior to injection. Activity counts were recorded during these sessions. Ten recipients were injected with pooled extract from the brains of ten control donors, ten were injected with saline solution, ten with pooled extract from trained rats, and ten with individual extracts, each from a separate donor. Two unreinforced test sessions (15-min) were given at 4 hr and 12 hr after injection. (A third session, with reinforcement, was added later, but the data considered here for this and all following experiments will be limited to unreinforced tests.)

Experiment 4: The same equipment was used as in experiment 3. Subjects were 20 trained donors, 20 control donors, 50 experimental, and 50 control recipients. Donors were given 6 days of training (30-min sessions). Two 10-min preconditioning sessions were given to the recipients, who were starved for two days prior to injection. Three unreinforced tests of 10 min each were

then given. To accommodate the large number of rats, four identical Skinner boxes were run in parallel, with equal numbers of control and experimental rats in each box. Five experimental and five control extracts were employed, each being administered to a group of ten rats. The first of these was an RNA extract from the whole brains of ten trained donors and ten controls, prepared as before. The remaining ten trained rats and 10 controls were used to prepare four fractions, as follows. The brains were homogenized with 3 ml saline per brain, centrifuged 1 hr at 60,000 *g*, and the supernatant was separated, saving the particulate fraction for the last extract. Twenty vol of cold acetone were added to the liquid phase, which was left for a half hour and then centrifuged for 10 min at 60,000 *g*. The resulting precipitate was taken up in Tris buffer, and successive column fractionation with Sephadex G-25 and G-50 columns was used to obtain molecular weight fractions of 1,000–5,000, 5,000–10,000, and a heavy fraction over 10,000. Each of these fractions was precipitated with 10 vol of cold acetone; the precipitate was collected by filtering, and taken up in a volume of 1 ml saline per rat for injection. The fifth extract was obtained from the particulate phase left over from the initial centrifugation. This was treated with a 50:50 phenol-saline mixture, as in the RNA preparation, and an acetone precipitate obtained from the saline phase.

Experiments 5 and 6: Standard Skinner boxes were used for training, as above. In the test sessions, however, the boxes were modified by the addition of a steel nut on a wire, with an automatic counter to record the number of times the nut was pulled by a rat. In experiment 6, this nut was available during preconditioning as well as testing. In addition to the bar-pressing count, a discrimination score (% bar-pressing responses out of total bar and nut activity) was obtained for each rat. Donors were ten untrained rats, ten rats trained for 5 days (30 min per day), ten trained for ten days, and ten rats trained for 15 days. Training sessions were reduced to 15 min after the first 5 days. Learning curves seemed to approach an asymptotic performance in about 10 days. Preconditioning (two 10-min sessions for expt. 5, and 3 for expt. 6) was given to a total of 140 rats, 12 of which were discarded from the sample so as to eliminate high and low extremes of spontaneous activity, and equalize activity rates of the various groups as far as possible. This left 124 recipients (64 in each experiment) who were divided into 16 groups of 8 rats each. Four different extracts were obtained from each of the four donor groups, each extract being injected into eight rats. Each group of eight was further divided into halves, four rats receiving a dose equivalent to 1.4 donor brains, and the other four rats receiving a dose equivalent to 0.7 donor brains. Four 10-min test sessions were run, over 2 days, beginning 10 hr after injection. The four extracts used were: (*A*), the molecular weight fraction from 1,000 to 5,000, as in experiment 4, but precipitated with only 10 vol of acetone; (*B*), the molecular weight fraction over 5000; (*C*), an extract from the particulate fraction, similar to the last extract in experiment 4 but including the phenol as well as the saline phase, and precipitated with ethanol to remove as much phenol as possible; (*D*), a suspension of the residual particle fraction left over from (*C*). Extracts (*A*) and (*C*) were injected in experiment 5, and (*B*) and (*D*) in experiment 6. One rat died during testing, and her data were not used. In analyzing the data from these experiments, the 0-day and 5-day groups (which were quite similar in performance) are treated jointly as "controls," with the 10-day and 15-day groups being treated as "experimentals."

Experiment 7: An automatically recording shuttle box was constructed by mounting a box with an electrified grid floor on a knife edge, and balanced at the center. A rat running to either side of the box would activate a counter, recording either the number of seconds spent on that side, or the number of crossings. The box was equipped with a light at each end and a buzzer. Rats were kept on a normal diet, shock being used for reinforcement. Sixteen rats were trained to stay on one side of the box to avoid a shock which was permanently connected to the "wrong" side. Eight of these rats were trained to stay on the light side of the box, and eight to stay on the dark side. Scores for these rats were based on the percentage of time spent on the dark side, the rats who were trained to stay on the light side being classified as "controls." A second group of 16 rats was trained to avoid a shock by crossing the box within 2 sec of a buzzer and light signal. Four counters were used to record the number of stimuli, the total number of times the rat crossed the box, the number of correct responses, and the number of "delayed responses" (within 4 sec of the warning signal). Stimuli were administered automatically at intervals of about 40 sec. The "fixed-shock" group was given nine training sessions of 15 min, rapidly approaching 100% of time spent on the proper side. The group trained to cross on the buzzer was given nine 30-min sessions, with somewhat poorer performance. Three of these rats, who learned to avoid the shock by standing on a

single grid bar, were eliminated from the sample. Extracts were prepared by homogenizing the brains in a phenol-saline mixture, and collecting both the phenol and saline phases and the interface material between them, which was avoided in earlier preparations. Two ethanol washes were found to be necessary to remove the phenol from this extract. The resulting material was injected into eight recipients for each of the first two groups of donors, 14 recipients for the buzzer-trained group, plus 14 controls who received a corresponding extract from untrained donors. For each group of trained donors, half of the brains were pooled and half extracted individually, as in experiment 3. Recipients were given two preconditioning sessions and six unreinforced test sessions (10 min per session for the "fixed-shock" group, or ten stimuli for the "buzzer" group). These tests, administered over a 3-day period, were followed by a second injection consisting of the residual particle fraction from the donors. Data from six more test sessions were then accumulated.

Experiments 8, 9, and 10: The training box used for these experiments was a modified Skinner box with a lever, food cup, and signal light at each end. Automatic counters were installed for each lever, and an automatic timing system was available for reversing the "effective" end and signal light. Sixteen rats were trained with the light in a constant position, eight of them being trained to operate the lever at the dark end, while eight "controls" were trained to operate the lever at the light end. Sixteen other rats were trained with the light alternating every half min, eight being trained to go to the light and eight to the dark. The per cent of responses on the dark side was used as a score for each rat. The first group (trained for 10 days) reached an asymptote of about 98% correct in 6 days. The alternating task was more difficult, the rats barely reaching a criterion of 90% correct after 10 days. In this, as in all other experiments with light signals, the rats showed a definite tendency to favor the light side of the box. Three rats were lost from the trained group (due to enteritis) before extracts could be obtained. The brain extract for experiment 8 was obtained by homogenizing the brains in a 50:50 phenol-saline mixture, and by ethanol precipitation from the combined saline and phenol phases. The residual particle fraction was saved for experiments 9 and 10. The extract from each group of eight rats was injected into eight recipients, without preconditioning.[20] Recipients were run for four unreinforced sessions, beginning 9 hr after injection. For experiment 9, the residual particle fractions were each divided into halves. One half was incubated with trypsin, at room temperature, for 30 hr at pH 8.5, and the second half was incubated with chymotrypsin under the same conditions. The liquid phase from these preparations was precipitated with acetone. The precipitate was suspended in saline and injected into 32 recipient rats. These rats were each given five 10-min preconditioning sessions, followed by five test sessions after injection. For experiment 10, the remaining particle fraction from the extracts of experiment 9 was pulverized ultrasonically, centrifuged 3 min at 2100 *g*, and the remaining suspension injected into 32 rats, who were given three preconditioning sessions and five test sessions.

Results.—Considerable differences in spontaneous activity rates, measured in preconditioning sessions, were observed. In order to control for this variation in activity, and to obtain scores which would facilitate the comparison of groups tested on a variety of different tasks under varying conditions, the following procedure was employed for analyzing the data: a linear regression equation was computed, based on the data of the control group in each experiment, to predict post-injection scores from preinjection scores (measured during preconditioning). The difference between the observed and predicted score (*O-P*) was computed for each rat, and this difference was then normalized by dividing it by the standard deviation of the *O-P* scores for the corresponding control group. This results in a standard score for each rat, normalized so that the mean score for the control group will always be zero, with unit standard deviation. The potency of transfer effects in different groups can then be expressed on a comparable basis in terms of the mean standard score of the group. A positive standard score indicates a performance above that of the control group, and a negative score represents a performance below that of the controls, measured in units of standard deviations. Whenever insufficient data are available to make use of the regression technique (as in expts.

1 and 2), the mean score of the corresponding control group is used as the predicted value, and the *O-P* scores are divided by the standard deviation, as usual. A third variation of this technique was employed in experiment 7, where a simultaneous activity measure was available for the rats trained to run on a buzzer signal. In this case, the total activity count (exclusive of responses to signals) was used to obtain predicted response rates by means of a regression equation, although an analysis based on preconditioning activity yielded essentially identical results.[21]

A frequency distribution of the standard scores for all control rats is shown in Figure 1, together with a frequency distribution for the standard scores of the experimental rats. Note that the lower half of each distribution is nearly identical, except for three extremely low scores for the experimental group; all of these came from experiment 9 where several rats appear to have acquired a strong tendency to press the bar, but only on the light side of the box, which is favored by most rats but is "wrong" for the experimental group. The upper halves of the distributions, however, reveal an extended "tail" for the experimental group, with a maximum score 22 standard deviations above the control group mean (obtained from one of the particulate extracts). A Mann Whitney *U*-test[22] can be used for a nonparametric estimate of the reliability of the difference between these two distributions. This shows that the superiority of the pooled experimental data over the pooled control data is significant at $P = 0.0006$. If the bottom 50 per cent of the experimental group is compared with the bottom 50 per cent of the control group, the experimental group is still found to be superior, despite the apparent similarity of the distribution curves in this region, with $P = 0.0066$. On the other hand, if the top half of the experimental distribution is compared with the top half of the control distribution, the difference is significant at $P = 1.5 \times 10^{-9}$. This suggests that the top half of the rats in each group should be compared for a particularly sensitive measure of the differences between experimental and control groups.

In our previous paper,[18] we have noted that the transfer effect seems to "take" on some rats, while leaving others unaffected. In the present series of experiments, we find repeated examples of this phenomenon, which is shown most dramatically in the difference between the top and bottom halves of the distributions. In cases where a successful transfer seems to have occurred, the experimental group generally has a number of extremely high scores, below which is a middle region consisting predominantly of controls, and at the bottom an indistinguishable mixture of control and experimental scores. The reasons for this bottom group, consisting of rats who apparently have no tendency to give the appropriate response, are still subject to speculation. At least some of these rats appear to be suffering adverse aftereffects from the injection (including injuries and phenol poisoning in several cases). Other contributing factors may be variations in dosage and site of injection, variations in threshold or sensitivity to the extract, and possibly, the transfer of an adap-

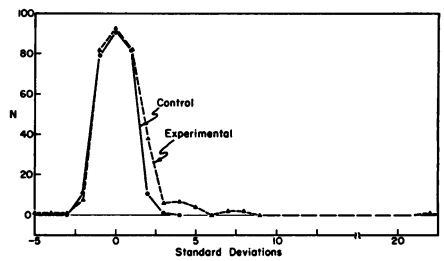

FIG. 1.—Frequency distributions of standard scores ($O\text{-}P/\sigma$) for combined experimental and combined control groups. Each point represents the number of scores in a class interval 1.0 standard deviations in width. Mean values are zero for the control distribution and 0.532 for the experimental distribution.

tation effect which tends to reduce activity that would otherwise result from normal curiosity. There are, indeed, several cases where low dosages or weak extracts seem to have an inverse effect of reducing rather than increasing the anticipated form of activity of the recipients, as in some of the nut/bar discrimination tests. In any case, the relative clarity of the effect for the "high rats" suggested that analyses should be run in parallel for the full samples and for the top 50 per cent of each group. Significance figures obtained by both of these methods will be presented in detail in the following paper.

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[20] Preconditioning was omitted in expt. 8 to see whether the rats might not respond better in the absence of previous adaptation. Subsequent experience has indicated that this is generally most undesirable, however, as the extra activity of the control animals tends to obscure the desired effect. Thorough adaptation seems to be a prerequisite for clear-cut results.

[21] Analyses done on the raw data, without controlling for activity, have yielded results qualitatively similar to those reported here. Since space limitations preclude a presentation of all of the raw data, only the final analyses are presented in this and the following paper. The following numbers, however, will help in establishing the relationship of the derived scores to the data from which they were obtained. The pooled data for all recipient rats tested in a single-bar Skinner box (expts. 3–6), excluding only the saline-injected group from expt. 3, come from 134 experimental and 123 control rats. The mean postinjection bar-pressing rates (in responses per min) were 0.425 for the experimental group and 0.369 for the controls. The range of the experimental group was from 0.03 to 3.23, and the control group ranged from 0 to 2.97. The probability of the difference in the means (by a U test) is 0.078. For the top 50% of the scores, the mean experimental rate is 0.711, and the mean of the top 50% of the controls is 0.592. The probability of this difference is 0.006. If the fraction with molecular weights over 10,000, and dosages less than one brain per rat are excluded from the sample (leaving 92 experimental and 82

control rats), the mean rate of the experimental group is 0.472, and the mean control rate is 0.372 ($P = 0.032$). For the top 50%, the mean experimental rate is 0.765, and the mean control rate is 0.606, with $P = 0.0025$. Response rates for expts. 1 and 2 have been summarized in our previous paper.[18] Nut-pulling response rates, in expts. 5 and 6, have a range from 0 to 3.25 responses per min, with percentages of bar-pressing activity ranging from 6 to 100% of combined responses. Response rates prior to injection tend to be 2–3 times higher than postinjection rates. In the shuttle box (expt. 7) the mean experimental response rate was 25.3 responses, and the mean control rate was 19.4 responses (within 4 sec) out of 120 stimuli, with a range from 12 to 38 responses in individual rats. About 90% of these responses were made during the 2 sec that the buzzer was sounding. The mean total activity rate for this experiment was 169 box-crossings for the experimental rats and 155 for the controls.

While results obtained with large numbers of rats using raw score data are similar to results from corrected data, consistent results from small samples seem to require careful control for spontaneous activity levels. For example, in expt. 5, the soluble fraction with molecular weights 1000–5000 with dosages of 1.4 brains per rat (eight experimental and eight control rats) has a mean response rate of 0.394 for the experimental group, and 0.263 for the control group. This difference is only significant at $P = 0.128$. Mean preinjection rates were 1.03 for the experimentals and 1.17 for the controls. The regression equation computed from the control data gave a predicted response rate of $0.177\ R_0 + 0.055$, where R_0 is the preinjection rate, for each rat. This gives mean predicted scores of 0.236 for the experimental group and 0.263 for the control group, with an advantage for the experimental group's *O-P* scores which is significant at $P = 0.002$. The obscuring of marginal effects by spontaneous activity, where this has not been adqequately controlled, may account for the seemingly negative results of many attempts at replicating the work of Babich *et al.*, which have been reported to us in private communications. It should be emphasized, however, that we have in no case succeeded in replicating the magnitude of the effect which they have obtained.

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*STAGES OF MEMORY FORMATION IN GOLDFISH: EVIDENCE FOR AN ENVIRONMENTAL TRIGGER**

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Goldfish given 20 shock-avoidance trials in a 40-min session show an increase in avoidance responses during the session.[1] Their performance further improves when they are given ten retraining trials 3 days later. We infer that the modifications in behavior are the result of changes in the brain which begin during the training session. The term "memory" is used to describe the alterations in the brain which are responsible for the increased responding.

Memory of the response after training can be obliterated by an intracranial injection of puromycin,[1–3] an antibiotic which suppresses protein synthesis *in vivo*.[4] The behavioral effect of puromycin suggests that the memory is formed in several stages. Fish given 170 μg of puromycin immediately following the training session perform 3 days later as though they are naïve.[2] When the interval between the last trial and the injection of puromycin is increased to 1 hr or more, however, responses 3 days later indicate that memory has been preserved. The evidence that puromycin causes a memory loss when injected within an hour following training,